Zn_2Mg Alkaline Phosphatase in an Early Ptolemeic Mummy

Yoka Kaup[a], Ursula Baumer[b], Johann Koller[b], Robert E. M. Hedges[c], Herbert Werner[d], Hans-Jürgen Hartmann[a], Hedwig Etspüler[a] and Ulrich Weser[a]

[a] Anorganische Biochemie, Physiologisch-Chemisches Institut der Universität Tübingen, Hoppe-Seyler-Straße 4, D-72076 Tübingen, Bundesrepublik Deutschland
[b] Doerner-Institut, Barer Straße 29, D-80799 München, Bundesrepublik Deutschland
[c] Research Laboratory for Archaeology and the History of Art, Oxford University, 6 Keble Road, Oxford, OX1 3QJ, U.K.
[d] Hygiene-Institut, Medizinische Mikrobiologie der Universität Tübingen, Silcherstraße 7, D-72076 Tübingen, Bundesrepublik Deutschland

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Bone samples of a ptolemeic mummy have been employed to study the mode of conservation on the intactness of Zn_2Mg alkaline phosphatase in both structure and catalytic activity. A protein of M_r = 190 ± 10 kDa being identical to the 200 kDa enzyme of fresh human bones was successfully isolated. Regardless of age 200 kDa protein bands and a distinct subunit at 60 kDa were seen in SDS-PAGE electrophoresis. The 200 kDa band was also monitored by activity staining. The specific activity was 120 mU/mg and 65% of the respective activity obtained in the identical preparation using fresh human tibia or rib. The enzymic activity was inhibited in the presence of 1,10-phenanthroline and L-homoarginine. Radiocarbon dating supported the assignment of the mummy to the early ptolemeic period. Among the many bactericidal and fungicidal components employed for mummification were aromatic alcohols, mono- and sesquiterpenes. Pistachio resin was the major balm resin used. The microbiological sterility of the bone surface was ascertained by independent bacterial and fungal examinations.

Introduction

Ancient conservation techniques led to surprisingly well preserved large M_r biopolymers including nucleic acids and proteins (Cano *et al.,* 1993; Tuross and Stathopolos, 1993; Weser *et al.,* 1989, 1990). Apart from the exceedingly good conditions the molecular intactness should likewise be reflected in the respective biological function of the examined component. Studies on structure function relationships of mummified proteins, however, are limited. An encouraging first result was the successful isolation of a small and enzymatically active remnant of Cu_2Zn_2 superoxide dismutase employing air-dried mummified brain tissue (Weser *et al.,* 1989, 1990). The cleavage of this 32 kDa protein into smaller fractions, though catalytically active, was thought to be attributed to the well pronounced oxidative strength of the coordinated copper. Metalloenzymes containing non-oxidation reduction active metals were thought to have a better chance to survive the conservation process unharmed. Furthermore, metalloenzymes in general do resist much more effectively uncontrolled proteolysis. In this context our choice fell on well characterized zinc enzymes which should be the ideal candidates for survival in the course of mummification.

During the New Kingdom and the Ptolemeic age mummies were pretreated with many different resins and ointments. Indeed, from skeletal muscles of these types of mummies considerable portions of high M_r proteins were obtained (Barraco, 1978). Unfortunately, no detectable functional activity was seen. It was attempted to elucidate the chemical nature of conserving agents and to search for concealed compartments where the balsamic resins did not reach intact enzymes. Bone tissues appeared to be the most promising source for a substantially diminished uptake of such resins. Another beneficial aspect for the conservation of proteins was the richly abundant hydroxyl apatite which was thought to serve as a most effective "ion exchange matrix" for the efficient binding of intact and catalytically active pro-

Reprint requests to Prof. U. Weser.
Facsimile: (+49) 7071 29 63 91.

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teins. Morphologically intact albumin and large portions of polynucleotides have already been isolated (Tuross and Stathopolos, 1993; Barraco, 1978; Pääbo, 1989; Sykes, 1991; Hagelberg *et al.*, 1989). However, limited data on the functional side are available.

It was of interest to improve these studies on a well characterized bone zinc enzyme. In this context Zn_2Mg alkaline phosphatase, known to be richly abundant in bones, was chosen to examine both intactness and catalytic function in a well preserved ptolemeic mummy. Prior radiocarbon assay of the mummified bones should ascertain the exact age. Gas chromatographic and mass spectrometric analyses of tissue extracts were performed to elucidate the chemical nature of the balm components and to determine compounds of antibiotic and/or antifungal activity. Direct microbial and fungal assays of the bone surface should describe the state of "sterility". Zn_2Mg alkaline phosphatase was isolated from bone tissue homogenates employing different chromatographic methods. The enzyme was examined for intactness using SDS microgel electrophoresis. The functional side was controlled employing the established *p*-nitrophenyl phosphate assay, and, in the course of SDS gel electrophoresis activity stainings were correlated with the respective protein bands.

Experimental

Materials

Deionized water was purified in a water purification system (Millipore, Eschborn). The conductivity was less than 0.05 μS. Superdex 200 prep grade HiLoad™ 16/60 columns, Sephadex G-25, Phast Gel Homogeneous 7.5 separation gels and PAGE buffer strips for a Pharmacia Phast System flat bed unit were obtained from Pharmacia LKB, Uppsala, Sweden. Bone alkaline phosphatase (Lot # 314) was obtained from CALZYME, San Luis Obispo, CA, U.S.A. The molecular mass marker (high M_r standard kit), *p*-nitrophenyl phosphate, 4-benzoyl amino-2,5-dimethoxy benzene diazonium chloride hemi [zinc chloride] salt (Fast Blue RR salt) and 3-hydroxy-2-naphthoic acid-2,4-dimethyl anilide phosphate (naphthol AS-MX phosphate) were from SIGMA, St. Louis, MO, U.S.A., Triton X-100 and diethanol amine from MERCK, Darmstadt. An Amicon Diaflo YM-10 (exclusion M_r = 10 kDa) ultrafiltration membrane, Amicon GmbH, Witten/Ruhr, Germany and a Microsep 10 K and 50 K (exclusion M_r = 10 and 50 kDa) centrifugal ultrafilt ration membrane, FILTRON, Northborough, MA, U.S.A. were used for protein concentration. Tris acetate buffer (0.01 – 1 M) was used throughout this study unless otherwise indicated. All other reagents employed were of analytical grade quality.

Bone preparations

Fresh human bone samples were obtained from the Berufsgenossenschaftliche Unfallklinik, Tübingen and the Pathologisches Institut der Universität Tübingen. The torso of a well preserved early ptolemeic mummy was kindly donated by the Staatliche Antikensammlung, München. A sample of a human femur bone, Abydos, 30th Dynasty, was a gift from the British Museum, London. All titanium tools were exclusively used to cut bone segments into smaller portions. A metallic titanium bar was generously given by IME-Titanium GmbH, Frankfurt. Titanium was selected as possible contaminating reactive metals including iron, cobalt, nickel and chromium were thus omitted to cause possible undesired oxidative degradation of biopolymers.

Radiocarbon dating

A radiocarbon date was measured using accelerator mass spectrometry (AMS) on a ptolemeic bone sample. In general, details of the chemical pretreatment, target preparation and AMS measurement are to be found in (Hedges *et al.*, 1989, 1992). For this particular bone, a rib sample of approximately 3 g was extensively extracted with acetone and then chloroform by sonication until constant weight. Collagen was extracted as insoluble protein after first washing the bone sequentially in diluted HCl, diluted NaOH and diluted HCl, followed by decalcification in 0.5 M HCl. The collagen was solubilized as gelatin, and the gelatin purified by ion exchange chromatography. The final yield of purified protein was approximately 50 – 100 mg protein per gram of bone indicating comparatively little degradation or loss of the protein. The purified gelatin was combusted to CO_2 which was measured for its stable isotope ratio. This was $\delta^{13}C = -17.5\%$ (relative to PDB); a value

consistent with a typical diet based on C3 and C4 vegetation. This implies that the purified gelatin is at least not grossly contaminated by carbonaceous material used in the mummification process which would have more -ve values for $\delta^{13}C$.

Microbial assay of bones

3.5 g bone were minced and ground under sterile conditions and suspended in brain heart infusion broth. Equal volumes of the suspension were inoculated to a) brain heart infusion broth and incubated under anaerobic conditions at 35 °C, b) brain heart infusion broth and incubated aerobically at 30 °C, c) Sabouraud dextrose broth and incubated aerobically at 25 °C. After 2 and 7 days incubation, subcultures from these media were made to the several solid media supporting growth of aerobic and anaerobic bacteria and fungi (Balows *et al.*, 1991).

Elucidation of mummification components

3.4 g of mummified skeletal muscle tissue was extracted in 500 ml 25% methanol/75% chloroform (v/v) for 9 h at 23 °C. The extract was concentrated at room temperature to 0.4% of the original volume. Aliquots containing 10% (v/v) of the tissue extract in the respective solution were redissolved in 2 ml methanol, chloroform and acidified methanol with 5% (w/v) oxalic acid, respectively. The respective extracts were injected without prior derivatization into the gas chromatograph as described in Koller and Baumer (1993). Gas chromatographic measurements were performed on a Hewlett-Packard unit HP 5890, Series II. For separation a 15 m fused J & W silica capillary column (DB 5-HT) was used with an inner diameter of 0.32 mm and a film thickness of 0.1 μm. The injection was carried out in the split mode at 270 °C. The detection temperature was 370 °C. Helium 5.0 served as the carrier gas at a total flow rate of 45 ml/min. The initial column pressure was 40 kPa. The temperature programmed GC run was started at $T_1 = 80$ °C. It was held for 2 min and then heated at a rate of $R =$ 10 °C/min. The final temperature was 360 °C. The gas chromatographic/mass spectrometric measurements were run on a Hewlett-Packard GC/MS system HP 5985 B.

Preparation of fresh and mummified bone Zn_2Mg alkaline phosphatase

All steps were performed at 4 °C. Large bone pieces obtained from rib, femur and pelvis were sawn using all titanium tools into smaller fragments and were finely ground in a porcelain mortar. 1 g aliquots each were suspended in 3 ml 20 mM Tris acetate (pH 7.4) containing 1 mM magnesium acetate, 0.3% (v/v) Triton X-100 and protease inhibitors (100 mM 6-aminohexanoic acid, 0.5 mM phenylmethyl sulfonylfluoride, 5 mM N-ethylmaleimide). Gentle agitation was maintained in the course of the extraction process. Fresh human bone samples were usually extracted for 3 h, the mummified species were allowed to rehydrate for 10 ± 2 h. The suspensions were centrifuged 30 min at 18,000×*g* and the supernatant was concentrated 20-fold by ultrafiltration through a YM-10 membrane. The protein concentration was determined spectrophotometrically at 595 nm following the Bradford technique (Bradford, 1976). Bovine serum albumin was used as a standard. Possible inhibitors including free amino acids were separated by passing the concentrated supernatant through a Sephadex G-25 column (1 cm×12 cm), equilibrated with 10 mM Tris acetate (pH 7.4), 2 mM magnesium acetate and 0.3% (v/v) Triton X-100 and eluted with the same buffer. 1 ml fractions were collected and assayed for alkaline phosphatase activity at pH 9.6 and 23 °C using *p*-nitrophenyl phosphate as the substrate (Magnusson *et al.*, 1993). The fractions containing active alkaline phosphatase were pooled and concentrated 4-fold by centrifugal ultrafiltration through a Microsep 50 K membrane. The concentrate was applied onto a HiLoad 16/60 Superdex 200 prep grade column previously equilibrated with 20 mM Tris acetate (pH 7.4), 2 mM magnesium acetate and 0.1% (v/v) Triton X-100. The elution was performed with the same buffer at a flow rate of 1 ml/min and controlled at 280 nm. 2 ml fractions were collected and each assayed for alkaline phosphatase activity and protein concentration.

Alkaline phosphatase assays

Enzymic activity of alkaline phosphatase was assayed in solution with *p*-nitrophenyl phosphate as substrate at pH 9.6 according to Magnusson *et al.* (1993). Activity staining of SDS gels after

electrophoresis was performed with 3-hydroxy-2-naphthoic acid-2,4-dimethyl anilide phosphate (naphthol AS-MX phosphate) at pH 8.0 as given in Spanos and Hübscher (1983).

The enzyme assay in solution was based on the increase of *p*-nitrophenol formation as a result of *p*-nitrophenyl phosphate hydrolysis caused by alkaline phosphatase recorded on a Beckmann DU 7400 spectrometer at 410 nm for a period of 60 min against a blank containing the solution buffer and the buffered substrate at 23 °C. 0.5 ml of the assay volume contained 50–100 µl aqueous enzyme and 400–450 µl substrate solution composed of 1.8 mM *p*-nitrophenyl phosphate and 0.5 mM magnesium acetate in 1 M diethanolamine, pH 9.6. One enzyme unit (U) is defined as 1 µmol substrate hydrolysis per minute. An extinction coefficient of $\in_{410} = 1.84 \times 10^4\ M^{-1}\ cm^{-1}$ was used.

The alkaline phosphatase activity in SDS gels was proportional to the formation of an azo-pigment. After SDS gel electrophoresis at 4 °C the gel was gently washed 3 times in 50 mM Tris·HCl, pH 7.5 for 30 min at 4 °C. The enzymic activity staining was then carried out in the dark at room temperature in 80 mM Tris·HCl (pH 8.0) containing 1.6 mg 4-benzoyl amino-2,5-dimethoxy benzene diazonium chloride hemi [zinc chloride] salt (Fast Blue RR salt) and 1.0 mg 3-hydroxy-2-naphthoic acid-2,4-dimethyl anilide phosphate (naphthol AS-MX phosphate) per milliliter. The reaction was completed after 12 h.

Relative molecular mass determination

The relative molecular mass of mummified Zn_2Mg alkaline phosphatase was estimated employing 7.5% Laemmli SDS-PAGE (Laemmli, 1970) by comparison with the respective M_r of the reference proteins and commercially available bone alkaline phosphatase. Sample preparation was performed after precipitation from diluted solution (Wessel and Flügge, 1984). The pellet was solubilized in Laemmli buffer omitting mercaptoethanol to avoid disulphide cleavage and Zn(II) extrusion and incubated for 5 min at 95 °C. In the case of activity staining a 5-fold concentrated Laemmli buffer in the absence of mercaptoethanol was added to the extract and the sample was incubated for 1 h at 4 °C. PAGE was carried out in a Pharmacia Phast System flat bed unit according to the procedures described in the manual at 15 °C for protein staining and 4 °C for activity staining. A Phast Gel Homogeneous 7.5 with a stacking gel (5% (w/v)) and a separation gel (7.5% (w/v)) was used. The PAGE buffer system contained 0.112 M acetate and 0.112 M Tris (pH 6.5). SDS buffer strips were composed of 0.2 M tricine, 0.2 M Tris and 0.55% (w/v) SDS (pH 8.1). PAGE conditions were 10 mA constant current and 65 Vh. After electrophoresis the protein bands were visualized by silver staining (Heukeshoven and Dernick, 1985) or activity staining (Spanos and Hübscher, 1983) as described above. Alternatively, molecular mass measurements were performed by gel chromatography. A HiLoad 16/60 Superdex 200 prep grade column was calibrated using either protein standards of known M_r: blue dextrane (600 kDa), glutamate dehydrogenase (350 kDa), xanthine oxidase (283 kDa) and bovine serum albumin (66 kDa), egg albumin (45 kDa).

Results

Radiocarbon dating

The torso of a well preserved ptolemeic mummy was used in the present study. In order to ascertain the suggested age a radiocarbon date measurement was performed using accelerator mass spectrometry on a rib bone sample. The actual measurement was made on the isolated collagen which was solubilized as gelatin. Surprisingly, little degradation or loss of the protein was observed supporting the excellent conservation of the mummy. The date obtained was 2290 ± 65 BP (OXA-4410). This is expressed as radiocarbon years BP, and is corrected for isotopic fractionation. To derive a calendrical date it is necessary to apply the calibration for this period. The results obtained calibrates as follows: a range with 1 sigma (67%) probability includes: 400–206 BC; a range with 2 sigma (95%) probability includes: 516–170 BC. In other words, the radiocarbon dating agrees with the suggested ptolemeic period and can be assigned to the late hellenistic and/or early ptolemeic age.

Chemical nature of balm components used for mummification

In order to elucidate the different components used for the mummification process a methanolic

solution of the mummified skeletal muscle tissue extract was subjected to gas chromatography. No detectable difference in either gas chromatogram was seen when methanol was replaced by chloroform for extraction. All detectable components within a total retention time of 30 min are clearly seen (Fig. 1).

Liquid components appeared during the first 5 min while high viscosity species are eluted up to 9 min. Solid fractions appeared after that retention time. Within the first 5 min the monoterpenes are the most abundant species (Fig. 2). The sesquiterpene fraction can be assigned until 9 min retention time. At 18 min diterpenes are detectable. Palmitate (11.8 min), oleate (13.6) and stearate (13.7) originate from the lipid portions of the skeletal muscle. Between 18 and 27 min the fraction containing triterpenes can be noticed (Fig. 1 and enlarged section in Fig. 4). Within 21 and 27 min triterpenes resin components and between 19 and 21 min aromatized triterpenes are allocated. The

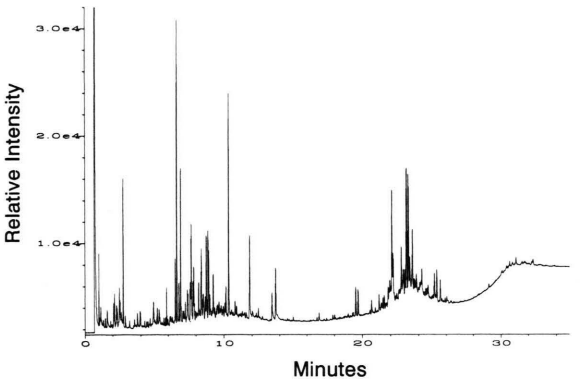

Fig. 1. Gas chromatogram of the balm materials extracted from mummified skeletal muscle. 3.4 g mummified muscle tissue was extracted in 500 ml 25% methanol/75% chloroform (v/v) for 9 h at 23 °C. The extract was concentrated at room temperature to 0.4% of the original volume. 0.2 ml of this concentrated extract was redissolved in 2 ml methanol and 1 µl of the resulting solution was injected without prior derivatization into a Hewlett-Packard gas chromatograph (HP 5890, Series II) fitted with split injection. Separation was done on a capillary column (0.32 mm i.d. × 15 m in length) of fused silica coated with a 0.1 µm thick layer of phenyl(5%)-methyl(95%)-siloxane (J&W, DB-5HT). The temperature was held at 80 °C for 2 min, then increased with 10 °C/min to 360 °C and held at this temperature for 5 min. Purified helium 5.0 was the carrier gas at a total flow of 45 ml/min and a column pressure of 40 kPa.

fractions beyond 28 min were outside the scope of the detection limit for GC/MS.

The liquid components appearing in the initial 5 min can be attributed to three major groups: monoterpenes, aromatic alcohols and derivatives of naphthalene. Among the group of monoterpenes α-pinene, cymene and limonene are found which are constituents of oil of turpentine obtained from the Allepo pine *(Pinus halepensis)* one of the oldest known oils. Carvacrol and verbenone (4-pinenone) are among the monoterpene alcohols and/or ketones. Likewise those fractions marked with question marks (monoterpenoids) have to be included. Considerable portions of aromatic alcohols have been elucidated, phenol, two isomeric forms of methyl phenol, three dimethyl phenol isomers and isopropyl cresol isothymol or carvacrol. Furthermore, methyl benzyl alcohol and xylyl ethanol were determined. In the third group naphthalenes are detected including naphthalene, methyl naphthalene and dimethyl naphthalene. They are predominantly originating from smouldering processes.

The extended section between 5 and 10 min retention time summarizes the sesquiterpene fractions (Fig. 3). Predominantly bicyclic hydrocarbons and their oxygen derivatives are seen. There are differences in the position and number of double bonds as well as the allocation of methyl and isopropyl side chains. Essentially three types of bicyclic C_{15} compounds containing two, three and four double bonds were assigned. Structurally related species include partially hydrogenated and alkylated derivatives of naphthalenes including isomeric forms of cadinenes. Tricyclic sesquiterpenes originating from azulene, for example, cedrene was clearly seen.

For a more specific assignment of diterpenoids acidic methanolic extracts were used. No diterpenes or diterpenoids, *e.g.* abietic and dehydroabietic acid as well as retene and tetrahydroretene were detected suggesting the absence of any pine resin, colophonium or wood tar derived from these resins.

Richly abundant were the triterpenoid fractions (Fig. 4). The peaks marked with M are the typical constituents of mastic resin which is plentiful available on the southern part of the island of Chios where the species *Pistacia lentiscus* is grown. They mainly belong to pentacyclic triterpenoids of

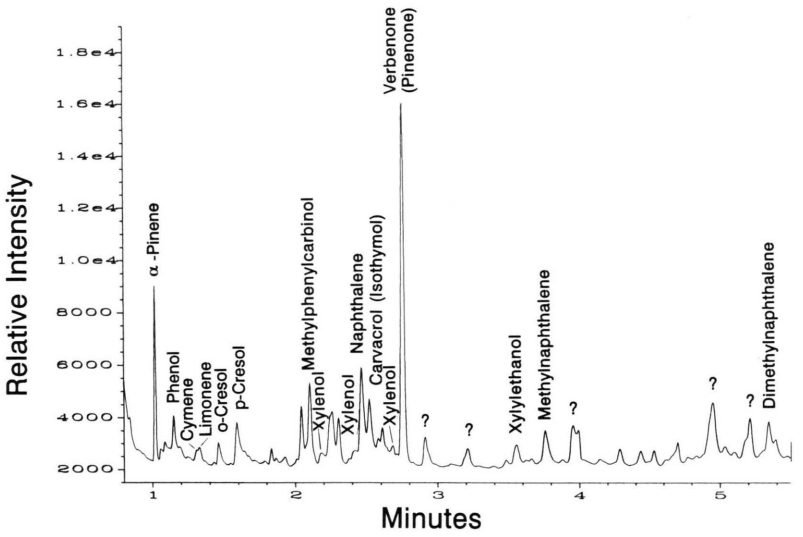

Fig. 2. Gas chromatogram of the liquid components of the mummy "tar". Enlarged section of Fig. 1 mainly containing the monoterpene fraction, the aromatic (phenolic) alcohols and the naphthalenes.

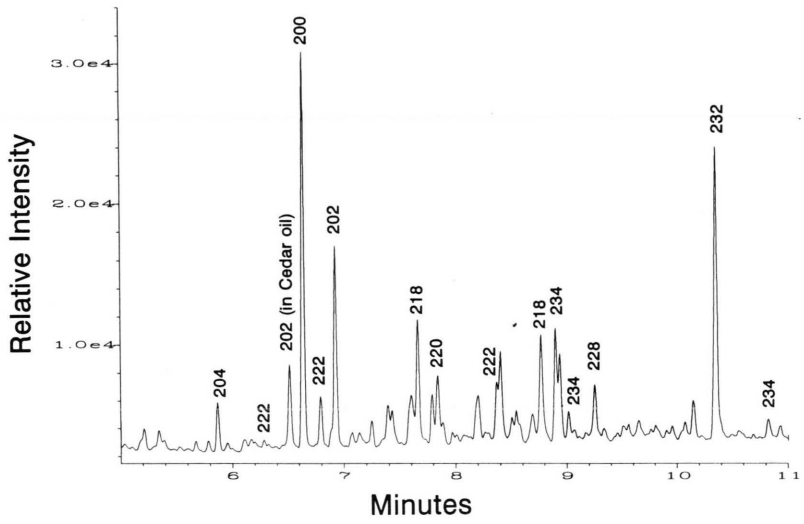

Fig. 3. Gas chromatogram of the high viscosity species of the mummy "tar". Enlarged section of Fig. 1 containing the sesquiterpene fraction. The numbers on the top of the peaks represent the molecular weights of the respective sesquiterpene components. The reference to cedar oil belongs to a commercially available product (Dragoco, Holzminden) which is not identical with the ancient cedar oil.

the oleanane series with noroleanone as the major component. The compounds emerging between the two main groups for mastic resin never have been isolated from mastic resin itself. They were rather found in dammar resin and gum elemi. These types of resins were certainly not used in the ptolemeic period. However, there was a close similarity to resins obtained from common pistachio. Due to the well known observation that pistachio trees secrete resins (Mills and White, 1987) the secrete of a branch of pistachio (Aegean island) was compared. The same fractions denoted M as before appeared but now together with peaks marked P. The virtual identity with the triterpenoid fraction of the mummy extract was most intriguing allowing the conclusion that in the course of balsaming the mummy pistachio resin must have been used. The components marked A

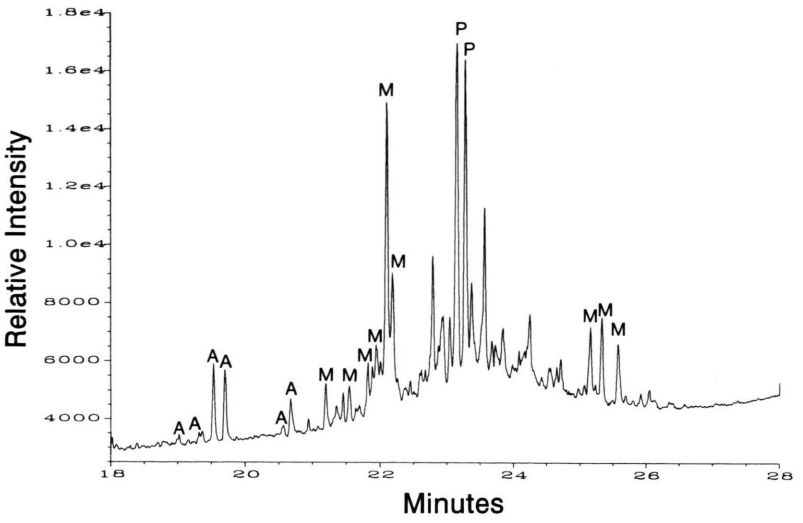

Fig. 4. Gas chromatogram of the resin fraction of mummy "tar". Enlarged section of Fig. 1 containing the triterpenoid fraction. The peaks marked with M are assigned as the constituents of mastic resin which is a special type of pistachio resin. Peaks marked with a P are additionally contained in common pistachio resins. The components marked A belong to aromatized or "skeleton" products based on triterpenoid type structure.

belong to aromatized and "skeleton" products based on triterpenoid type structure. In untreated pistachio resin these A fractions are absent. They might have been formed in the course of smouldering processes.

Microbial bone contamination

Alkaline phosphatase is likewise abundant in microorganisms. Although the relative molecular mass of these enzymes is significantly smaller compared to that of the vertebrate enzyme control experiments were carried out to examine possible microbial contamination of the bone surface. Mummified bone samples were minced under sterile conditions and suspended in brain heart infusion broth and incubated both anaerobically and aerobically. Additionally Sabouraud dextrose broth was used. Subcultures of the above media were applied to several solid media for up to 16 days. No anaerobic or aerobic bacteria or fungi could be detected suggesting the powerful bactericidal and fungicidal reactivity of the employed resin components used in the mummification process.

Isolation of bone Zn_2Mg alkaline phosphatase

Prior to the preparation of the mummified enzyme control extractions from fresh human bone samples became necessary. Bone fragments were homogenized in a porcelain mortar and pistill, suspended in Tris/Triton buffer and kept at 4 °C in the presence of protease inhibitors. The supernatant was membrane filtrated and chromatographed over Sephadex G-25. A specific activity of 10 mU/mg protein in the eluted exclusion volume was assayed. The eluates containing active portions of Zn_2Mg alkaline phosphatase were concentrated by centrifugal ultrafiltration and passed through a Superdex 200 column (Fig. 5).

The strong activity peak overlapping the protein band in the exclusion volume near 45 ml was assigned to the known phenomenon of phosphatase binding to Triton X-100 micelles forming oligomers which were detected in the exclusion volume presently at 600 kDa (Baileys *et al.*, 1987). The major and sharp protein band at 200 kDa overlaps a splitted activity peak with the major portion near 190 kDa. Four clearly separated peaks appear between 110 and 20 kDa. The peaks near 60 kDa can be assigned to a well characterized but inactive subunit (Weiss *et al.*, 1986) also prevalent in SDS gel electrophoresis. The highest specific activity in the 200 kDa peak was 180 mU/mg.

The same technique was applied to isolate Zn_2Mg alkaline phosphatase from mummified bones. Unlike the experiments with fresh bone the homogenized mummified rib sample in Tris/Triton buffer was allowed to extract 3–4 times longer. It was depressive to realize that no enzymic activity was detectable in the supernatant of the centrifuged suspension. As no significant balm compo-

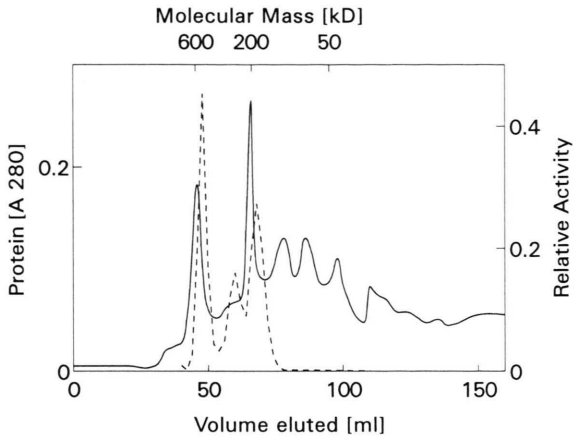

Fig. 5. Superdex 200 gel filtration of an extract of a fresh human bone. Protein (——), activity (– – –). 2–4 g finely ground bone fragments were suspended in 3 ml 20 mM Tris acetate (pH 7.4) containing 1 mM magnesium acetate, 0.3% (v/v) Triton X-100 and protease inhibitors (100 mM 6-aminohexanoic acid, 0.5 mM phenylmethylsulfonylfluoride, 5 mM N-ethylmaleimide) under slight agitation for 3 h at 4 °C. After centrifugation at 18,000×*g* the supernatant was concentrated 4-fold by ultrafiltration through a YM-10 membrane. Protein was quantitated at 595 nm (Bradford, 1976). Serum albumin was used as standard. 5 mg of the concentrated supernatant (5 mg/ml) were chromatographed on a HiLoad 16/60 Superdex 200 prep grade column previously equilibrated with 20 mM Tris acetate (pH 7.4), 2 mM magnesium acetate and 0.1% (v/v) Triton X-100. Elution with the same buffer was controlled at 280 nm. 2 ml fractions were collected and assayed for alkaline phosphatase activity at pH 9.6 and 23 °C. The assay mixture contained 1.8 mM *p*-nitrophenyl phosphate in 1 M diethanolamine and 0.5 mM magnesium acetate, pH 9.6. Formation of *p*-nitrophenol was estimated at 410 nm using $\in_{410} = 1.84\times10^4\ M^{-1}\ cm^{-1}$ (Magnusson *et al.*, 1993). For further details see experimental section.

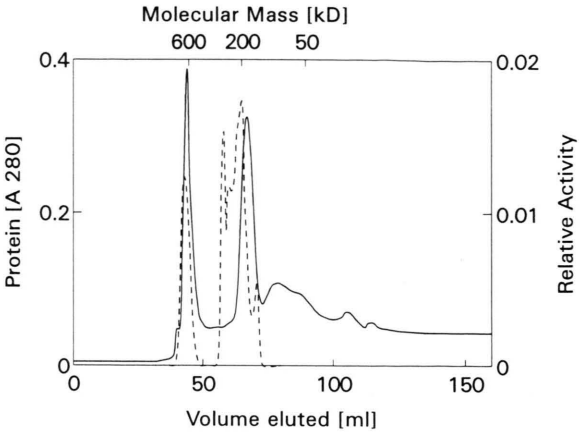

Fig. 6. Superdex 200 gel filtration of bone extract from an early ptolemeic mummy. Protein (——), activity (– – –). 4.6 g finely ground bone fragments of a ptolemeic mummy were extracted in 14 ml Tris/Triton buffer for 12 h. 0.05 mg of the 20-fold concentrated supernatant (0.05 mg/ml) were applied on a Superdex 200 column. All further steps were carried out exactly as described above.

nents were monitored this phenomenon was thought to be attributed to the presence of free amino acids or low M_r peptides known to be inhibitors of alkaline phosphatase. The latter species originated from the disintegration of some proteins, a consequence of the long storage. The separation from these possible inhibitors was successful employing membrane filtration and chromatography on Sephadex G-25. The specific activity in the separated exclusion volume was 24 ± 3 mU/mg. It was attempted to improve the separation of the enzymatically active protein portions employing Superdex 200 chromatography. It would be not unexpected to separate partially cleaved remnants of active alkaline phosphatase. However, it was astounding to realize the presence of large M_r proteins of considerable activity. In fact the elution pattern between 600 and 200 kDa was identical to that of the fresh bone isolation (Fig. 6).

Again the Triton X-100 micelles have trapped considerable portions of alkaline phosphatase at 600 kDa. In the 200 kDa region a splitted activity peak with some additional small spikes are seen. The specific activity was 120 ± 3 mU/mg being 65% of the activity in the fresh bone preparation. The distinct 4 peaks below 170 kDa were missing in this elution. In the light of the ageing process this phenomenon was not surprising.

The availability of fairly large bone samples in the ptolemeic mummy was quite fortunate for the successful chromatographic separation of intact Zn_2Mg alkaline phosphatase. In order to see the limitations of this isolation method related to sparingly bone probing some 200 mg of a femur bone sample obtained from a dated mummy 30th Dynasty, Abydos, were subjected to the same before mentioned protocol (Fig. 7).

After Superdex 200 chromatography the elution pattern was not too much different from the previous two versions. Due to the substantially diminished enzyme concentration 600 kDa Triton X-100 aggregates were absent. All activity was located

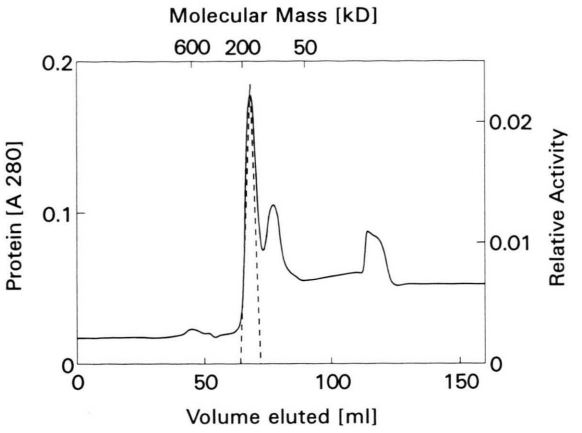

Fig. 7. Superdex 200 gel filtration of a femur bone extract (Abydos, 30th dynasty). Protein (——), activity (– – –). 0.2 g bone fragments of a ptolemeic female mummy were suspended as above in 0.6 ml Tris/Triton buffer for 12 h. 0.01 mg of the concentrated supernatant (0.01 mg/ml) were applied on the Superdex 200 column and processes in the same manner as stated in the legend to Fig. 1 and 2. 2 ml fractions were collected and assayed for alkaline phosphatase activity at 410 nm as described in the legend to Fig. 1.

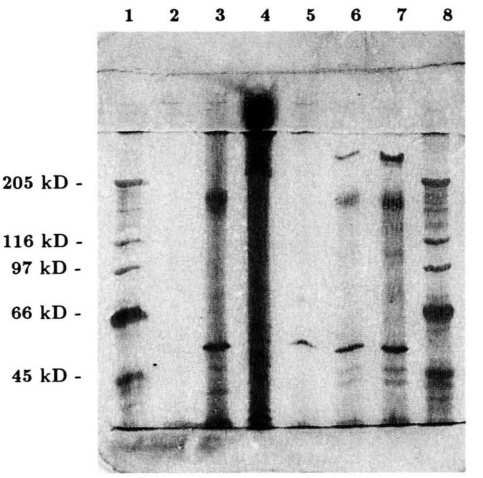

Fig. 8. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of alkaline phosphatases of different origin. (1) and (8) molecular mass marker myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa); (2), (3), (4) and (5) mummified enzyme, different preparations after gel filtration (3 μg); (6) commercial available alkaline phosphatase (0.5 μg); (7) commercial available alkaline phosphatase (0.8 μg). The relative molecular mass estimation of mummified Zn_2Mg alkaline phosphatase on 7.5% Laemmli SDS-PAGE was compared with those of the reference proteins and commercial available bone alkaline phosphatase (Calzyme, Lot # 314). The proteins were precipitated from diluted solutions and the pellet was solubilized in Laemmli buffer and incubated for 5 min at 95 °C. PAGE was performed in a Pharmacia Phast System flat bed unit which was programmed as given in the manual. A Phast Gel Homogeneous 7.5 with a stacking gel (5% (w/v)) and a separation gel (7.5% (w/v)) was used. PAGE was performed at 15 °C, 10 mA and 65 Vh. After electrophoresis the protein bands were visualized by silver staining (Heukeshoven and Dernick, 1985).

in the 190 kDa fraction. The concentration of low relative molecular mass compounds was higher. This was attributed to the conservation of the mummified bone at a lower quality. The specific activity rose to 154 mU/mg protein in the 190 kDa fraction some 34 mU higher compared to the enzyme fraction obtained from the ptolemeic torso. This elevated value can be assigned to the reduced overall protein content in the 190 kDa eluate.

It has to be taken into account that the chromatographed Zn_2Mg alkaline phosphatases on Superdex 200 coelutes with some proteins other than the present zinc enzyme. An alternative separation on SDS gel was promising to obtain additional proof of the intactness of the mummified enzyme. The Sephadex G-25 filtrated active protein fraction was applied on a SDS gel and compared with protein fractions isolated from fresh human bone and homogeneous Zn_2Mg alkaline phosphatase (Fig. 8).

Identical to the fresh human bone enzyme the mummified enzyme migrated at M_r = 200 kDa. In either electrophoresis and independent of the age of the employed protein a characteristic subunit appearing at 60 kDa was seen. This phenomenon is consistent with the reported 542 amino acid polypeptide chain of 57.2 kDa (Weiss *et al.*, 1986). The enzymic activity of the two distinct protein bands at 200 and 190 kDa was controlled by direct staining with an azo dye (Fig. 9).

The SDS electrophoresis pattern of samples of fresh human rib and commercially available Zn_2Mg alkaline phosphatase displayed two distinct activity spots each at 190 and 200 kDa. Unlike the splitted activity eluates obtained from the ptolemeic mummy employing Superdex 200 chromatography a splitting was not detectable in the SDS gel electrophoresis. Nevertheless, a sharp and well developed activity band was clearly seen. It was most intriguing to realize that the activity of

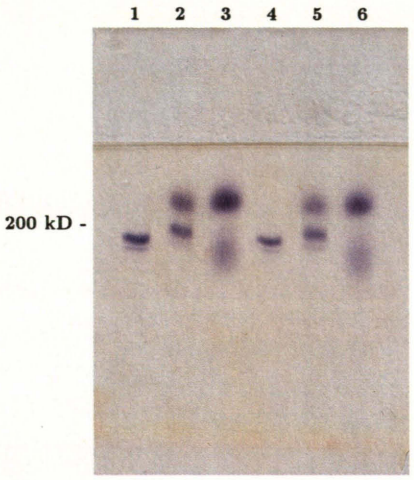

Fig. 9. Activity staining of mummified and fresh alkaline phosphatase on SDS gel. (1) and (4) mummified bone extract; (2) and (5) fresh human bone extract; (3) and (6) alkaline phosphatase (Calzyme, Lot # 314). The proteins were precipitated from diluted solutions. Either pellet was solubilized in 5-fold concentrated Laemmli buffer and incubated for 1 h at 4 °C. Electrophoresis of 0.8 µg protein each was performed at 4 °C as described above. After electrophoresis the alkaline phosphatase was visualized by activity staining using azo-pigment formation (Spanos and Hübscher, 1983).

the mummified enzyme was approximately half to that of the fresh homogeneous alkaline phosphatase. As in the gel filtration studies all 60 kDa subunits remained enzymically inactive.

Due to the richly abundant Zn(II) in the examined protein fractions an assignment of the exact zinc stoichiometry in the enzyme was not possible. Zn(II) displacement and/or inhibiting studies using Zn(II) chelators appeared to be more promising. The catalytic function of the enzyme was inhibited by 41% in the presence of a 2500-fold molar excess of Zn(II)-chelating 1,10-phenanthroline. The inhibition was 44% when the same molar excess of L-homoarginine a well known specific inhibitor of alkaline phosphatase was used. This inhibition was consistent with earlier studies using alkaline phosphatase from fresh human tissues (Lin and Fishman, 1972). These observations substantially support the conclusion that the zinc containing alkaline phosphatase was the active protein surviving 2300 years of mummification essentially unharmed in both structure and function.

Discussion

In the fast growing field of molecular archaeology a study on the mode of conservation of mummified tissues on the intactness of enzymes in both structure and catalytic activity has been performed. Initially it was of importance to support the archaeological date assignment of the mummified torso to the ptolemeic period. The radiocarbon date measurement was in perfect agreement with the former conclusion. It was intriguing to realize that little degradation or loss of the protein had occurred. The absence of carbonaceous carbon was indicative to the fact that no asphalt of fossile origin was used for mummification.

The major balm component proved to be pistachio resin which was suspended in a perfuming oil, possibly oil of cedar, and, initially a lot of oil of turpentine. A decision whether or not the aromatic alcohols found in the solvent fraction are attributable to an additional essential oil component and/or a by-product obtained in the case of harvesting natural resins remains to be elucidated. The mixture of phenol, phenol derivates, homologous forms of phenols such as cresol, xylenol and carvacrol are also found in crude origanum oils prepared in northern Africa and the Middle East. They are long known for their antiseptic properties (Anon., Schimmel & Co., 1938). Of course, phenolic compounds are formed in the processes of smouldering cedar wood chips. The evaporated mist containing phenolic compounds were condensed in loosely packed wool plugs at the top of amphorae. Naphthalenes, methyl- and dimethyl naphthalenes are concomitantly formed during this smouldering process usually devised for the preparation of wood tar oil. The final aromatic species found in the monoterpenes fraction convincingly support this conclusion together with the hydrogenated and alkylated naphthalene and azulene compounds of the sesquiterpene fraction.

All phenolic compounds including phenols, cresols, xylenols and carvacrols are highly reactive disinfectants. A pronounced anti-worm activity has been reported for carvacrol. All mono- and sesquiterpenes are highly fungistatic.

The many characterized balm components agree in a rather limited manner with the claimed resins and ointments described by Forbes (1965). Cassia,

cedar oil, other ointments, myrrh, cinnamon, other fragrant materials and cheaper, less efficient oils were used in the balsaming processes. Pistachio resin is not dealt with at all. It has to be emphasized that pistachio resin belongs to the most clearly identified component. In ancient times the use of pistachio resin was well known and the trade with this product was quite common. From a sunken ship considerable portions of this resin were obtained (Mills and White, 1989).

According to the many identified balm components it was not surprising to observe a virtual "sterility" of the dissected bone samples. Much to our surprise the bone surface acted like a barrier to minimize penetration of these reactive species into the matrix. The many different hydrophobic and reactive compounds remain dissolved in the balm resins and are rejected by this polar matrix. The ion exchange properties of hydroxyl apatite, most likely, can be assigned to the tenacious binding of the many charged phenolic and alcoholic species. The apatite density at the bone surface is especially strong and would explain the barrier phenomenon. The exceedingly good preservation of protein by hydroxyl apatite should be pointed out. When isolated Zn_2Mg alkaline phosphatase is kept at room temperature in aqueous buffers the enzymic activity is completely diminished in less than 2 h. By way of contrast, the bone bound enzyme survives heating at 55 °C for more than 10 days. This "mimicking" of mummification as well as the actual occurrence of structurally and functionally intact alkaline phosphatase in mummified bones demonstrates the usefulness of this enzyme as a potent marker for the elucidation of archaeological questions on a molecular basis. The dependency of mummification techniques, medical and nutritional status on this marker will be of utmost importance for further studies.

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